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Interpretive summary:

Microbial samples from several experiments in lactating dairy cattle were analyzed for nutrient composition, AA composition and digestibility. An in vitro assay was used to determine intestinal availability of individual AA. Multiple time point hydrolysis and least-squares non-linear regression was used to determine the AA content of microbial samples, and comparisons were made against single time point hydrolysis. Results indicated that the in vitro assay could demonstrate differences in AA digestibility, and multiple time point hydrolysis likely provide more accurate estimations of the AA profile of omasal bacteria and protozoa for use in field applicable nutrient models.

Ruminal bacteria and protozoa composition, digestibility and amino acid profile determined by multiple hydrolysis times.

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ABSTRACT: Microbial samples from 4 independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA digestibility, and AA profile after multiple hydrolysis times ranging from 2 to 168 h. Similar bacterial and protozoal isolation techniques were used for all isolations. Omasal bacteria and protozoa samples were analyzed for AA digestibility using a new in vitro technique. Multiple time point hydrolysis and least-squares non-linear regression were used to determine the AA content of omasal bacteria and protozoa, and equivalency comparisons were made against single time point hydrolysis. Formalin was used in 1 experiment, which negatively affected AA digestibility and likely limited the complete release

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of AA during acid hydrolysis. The mean AA digestibility was 87.8 and 81.6 % for non-formalin treated bacteria and protozoa, respectively. Preservation of microbe samples in formalin likely decreased recovery of several individual AA. Results from the multiple time point hydrolysis indicated that Ile, Val, and Met hydrolyzed at a slower rate compared with other essential AA. Singe time point hydrolysis was found to be non-equivalent to multiple time point hydrolysis when considering biologically important changes in estimated microbial AA profiles. Several AA including Met, Ile, and Val were under-predicted using AA determination after a single 24 h hydrolysis. Models for predicting post-ruminal supply of AA might need to consider potential bias present in post-ruminal AA flow literature when AA determinations are performed after single time point hydrolysis and when using formalin as a preservative for microbial samples.

Keywords: amino acid, digestibility, hydrolysis, ruminal bacteria, ruminal protozoa

INTRODUCTION

Nutrient supply and requirement models such as the Cornel Net Carbohydrate and Protein System (**CNCPS**) (Higgs et al., 2015; Van Amburgh et al., 2015) and the NRC (NRC, 2001) along with their derivative models predict post ruminal flows of bacterial biomass. Bacterial protein flow is assigned an AA content, AA profile, and a digestibility of individual AA to calculate supply of metabolizable AA. These field applicable models typically use the AA profile of bacteria obtained from the literature (Storm et al., 1983; Clark et al., 1992; Volden and Harstad, 1998), and few account for protozoal AA flows, which can contribute a substantial amount to total microbial AA flow (Dijkstra et al., 1998; Fessenden, 2016). A new, dynamic version of the CNCPS (v. 7; Higgs, 2014; Higgs et al. submitted) utilizes a similar approach in a N based model, and mechanistically accounts for protozoa and endogenous AA contributions to total AA flow in addition to bacteria and feed. To improve this model, accurate representations

of the AA content and digestibility of bacterial and protozoal AA were needed to understand sources of error in predictions of particular AA. On an N basis, the predictions of NAN in CNCPS v. 7 were reasonably accurate and precise. However, predictions of individual AA such as Lys, Met, Ile, and Val were biased, likely due to a lack of information about the true content those AA in both microbes and feeds (Higgs, 2014; Higgs et al., submitted).

The methods used for isolation of microbial fractions and analysis of AA vary widely across the literature, and much of the data used for nutrition models still rely on older methods where more robust alternatives now exist. An example can be found in the isolation of protozoa, where differential centrifugation has historically been used to isolate microbial cells with significant contamination from bacteria and feed particles. A procedure developed for isolation of cultivatable mixed ruminal protozoa for qPCR and competition studies (Sylvester et al., 2004; 2005; Denton et al., 2015) can be used to isolate protozoa for more accurate nutrient analysis. In addition, several of the methods employ the use of formalin in the isolation and storage of microbial and specifically protozoal samples (Martin et al., 1994; Rossi et al., 2013). This might be problematic as formaldehyde is known to react with AA (Barry, 1976).

Intestinal digestibility of individual microbial AA has been estimated by several different methods, including regression approaches (Tas et al., 1981; Hvelplund and Hesselholt, 1987), in vitro assays such as the modified 3-step assay (Gargallo et al., 2006), and in vivo assays utilizing the mobile bag technique. In vivo and in vitro procedures relying on retention in bags are largely inadequate, as both indigestible and digestible protein and AA can pass through the pores in the bags resulting in inflated digestibility values. In vivo assays have clear advantages in providing realistic and applicable enzymatic hydrolysis conditions, however they also require surgically altered animal models. The precision-fed cecectomized rooster bioassay was recently applied to

ruminal bacteria (Fonseca et al., 2014); however data are still lacking on protozoa AA digestibility. Additionally, avian species have different enzymes and pH conditions which might reduce the ability to make direct comparisons to ruminant digestion (Keller, 1968; Guerino and Baumrucker, 1987). Ross et al. (2013) developed an in vitro assay with ruminal fluid to determine intestinally unavailable N (**uN**) in ruminant feeds that addressed some issues of non-physiologic or species specific enzyme activities, poor retention of small particles, and extensive cannulation procedures. This assay might provide an adequate assessment of intestinal digestibility of ruminal bacteria and protozoa. Considering the ability of formaldehyde to reduce feed protein degradation, it is also possible that the use of formalin in preservation of microbial samples, as reported in Reynal et al. (2003) and others, might reduce the measured intestinal digestibility of microbial AA. An evaluation of the digestibility of unpreserved and formalin preserved samples using the uN assay might simultaneously provide more information on formalin effects on individual microbial AA while testing the ability of the uN assay to detect differences in samples treated with a compound known to decreased digestibility.

Amino acid content of feeds and microbes has historically been determined by single time point hydrolysis, as this represents a compromise between maximal release of AA from the matrix while minimizing the loss of acid labile AA (Rutherfurd, 2009). Determination of AA at multiple time points followed by least-squares non-linear regression provides more accurate estimates of the AA profile (Darragh and Moughan, 2005). This approach has been utilized in purified protein (Darragh et al., 1996), milk protein (Rutherfurd et al., 2008) and common animal feedstuffs (Rutherfurd, 2009). Previous work in our laboratory indicated that to obtain the greatest release of branched-chain AA in forages, hydrolysis times needed to be greater than 21

hr; while Ile release was greatest at 70 hr (Ross, 2004). To our knowledge, AA determination after multiple hydrolyses times has not been performed on rumen microbial biomass.

Given the data from Darragh and Moughan (2005), Rutherfurd (2009) and the observations made in the data from Higgs et al. (submitted), the hypothesis of this study was that the standard method of determination of AA in ruminal bacteria and protozoa using single time point hydrolysis is not equivalent to AA determination after multiple time point hydrolysis and non-linear least-squares regression. Additional objectives of this study were to summarize the chemical composition and AA profile of ruminal bacteria and protozoa from high producing lactating dairy cows and to evaluate intestinal digestibility of microbial AA using a newly developed in-vitro assay.

MATERIALS AND METHODS

All cannulated cows used as rumen or omasal fluid donors for the microbial isolations in this experiment were cared for according to the guidelines of the Institutional Animal Care and Use committee appropriate for the university responsible for their care. The committees reviewed and approved the experiment and all procedures carried out in the study.

Microbial Isolation Procedures

Microbial samples from several independent experiments in lactating dairy cattle were obtained and analyzed for nutrient composition, AA content, and intestinal nutrient and AA digestibility. Bacteria and protozoa included in the analysis were from the following experiments: **Trial A**: An omasal sampling trial with 8 cows in a 2 treatment switchback design investigating effects of a commercial byproduct feed on omasal nutrient flow (Fessenden, 2016);

Trial B: An omasal sampling trial with 12 cows in a 3 treatment Latin Square design investigating the effect of rapidly degradable starch on omasal nutrient flow (Foskolos et al, unpublished data); **Trial C**: A ruminal N balance and recycling trial with 12 cows in a 3 treatment randomized complete block design investigating ruminal N and/or MP deficient diets (Recktenwald, 2010; Recktenwald et al., 2013). One additional protozoal sample was obtained from T. Hackmann at the University of Florida from repeated isolations from the rumen of a lactating dairy cow at the Ohio State University Columbus campus **(Trial D)**. For trials A-C, equal parts DM were combined within microbial type, resulting in a composited sample of bacteria and protozoa from each experiment. Therefore, the possible effects of treatments from trials A – C are not represented in this dataset. Information regarding the chemical composition of the average diet fed to cows in each experiment, along with the number of individual collections and isolations represented by each composited sample are reported in Table 1. Due to limited amount of sample for some trials (D and C) not all analysis were performed on all samples as noted throughout the text.

For Trials A and B, microbial samples were obtained using the omasal sampling technique developed by Huhtanen et al. (1997) and adapted by Reynal and Broderick (2005). Samples of whole omasal contents were collected from the omasal canal every 2 h during 3, 8 h intervals. Details of sampling for trial A are described in Fessenden (2016). Trial B sampling occurred per a very similar sampling schedule as Trial A by the same researchers (A. Foskolos and S. Fessenden). Trial C collection methods are described in Recktenwald (2010) and Recktenwald et al. (2013). Trial D protozoa were collected on 4 separate days with 2 separate aliquots filtered per day for 8 aliquots total (T. Hackmann, personal communication).

Bacterial isolations for trial A and C were performed according to Whitehouse et al. (1994) with modifications. Briefly; whole omasal contents were filtered through 4 layers of cheesecloth and solids were rinsed once with saline, and the filtrate (I) was treated with formalin (0.1% v/v in final solution) and stored at 4 °C. The solids retained on the cheesecloth were incubated for 1 h at 39 °C in a 0.1% methylcellulose solution, mixed for 1 min at low speed (Omni Mixer, Omni International, Kennesaw, GA) to detach solids associated bacteria, and held at 4°C for 24 h. The contents were then squeezed through 4 layers of cheesecloth and the filtrate (II) was treated with formalin (0.1% v/v in final solution). Filtrates I and II were then combined and centrifuged at 1,000 x *g* for 5 min at 4 °C to remove small feed particles and protozoa. The supernatant was centrifuged at 15,000 x *g* for 20 min at 4 °C and the bacterial pellet, representing both solid and liquid associated bacteria, was collected and stored at −20 °C until lyophilization and later analysis. Bacterial isolation for trial B followed the same procedure as described above, however formalin was not used.

Protozoa from trials A and B were isolated from whole contents using the same procedure as described by Denton et al. (2015) and modified as reported in Fessenden (2016) (Figure 1). The only difference between trials was the omission of formalin and centrifugation in Trial B. The isolations were performed by the same researcher for both trials. Strained omasal fluid (250 mL) was combined 1:1 with pre-warmed, anaerobically prepared Simplex type buffer and added to a pre-warmed separatory funnel. Plant particles were removed by aspiration after 1 h of incubation at 39 °C. Funnel contents were then preserved with formalin (0.1% v/v in final solution) and stored for < 4 d at 4 °C. Preserved contents were centrifuged at 1000 x *g* for 5 min at 4 °C, the pellet was re-suspended in saline, and protozoa were isolated on a nylon cloth with a 20 µm pore size (14% open area, Sefar, Buffalo, NY). The protozoa isolate was washed several times with

158 saline (500 mL) to reduce bacterial contamination. After isolation, protozoa were stored at −20
159 °C prior to lyophilization. Protozoa from trial C were isolated from strained ruminal fluid by
160 flocculation to remove feed particles followed by preservation with formalin and centrifugation
161 at 500 x *g* for 5 min at 10°C. The pellet obtained after centrifugation was assumed to be
162 representative of the ruminal protozoa (Recktenwald, 2010). Protozoa isolation from Trial D was
163 performed by T. Hackmann in the laboratory of J. Firkins at the Ohio State University according
164 to Denton et al., (2015) except 25 mL instead of 30 mL or clarified fluid was filtered per
165 isolation.

166 ***Chemical Analysis and Hydrolysis Times***

167 All samples were analyzed for DM after 6 h at 105°C and ash according to AOAC (2005).
168 Total N was determined using a combustion assay (Leco FP-528 N Analyzer, Leco Corp., St.
169 Joseph, MI). Amino acid content of all samples was determined by HPLC following hydrolysis
170 for 24 h at 110°C in a block heater (Gehrke et al., 1985). Trial B bacteria and protozoa were also
171 hydrolyzed for 2, 4, 6, 12, 18, 21, 24, 30, 48, 72, 120 and 168 h to evaluate the rate of release of
172 each AA. The time points chosen were based on a similar analysis performed on milk proteins
173 (Rutherfurd et al., 2008). The entire time course was performed twice for each sample, and the
174 reported values are the mean of the 2 determinations. Insufficient sample amount from trials A,
175 C, and D precluded the multiple time point hydrolysis of samples for AA determination.

176 For all AA excluding Met, Cys, and Trp, a sample containing 2 mg N was weighed into
177 hydrolysis tubes with 25 µL of 250 m*M* norleucine as an internal standard. Samples were then
178 hydrolyzed as described above with high-purity 6 *M* HCl (5 mL) after flushing with N_2 gas
179 (Mason et al., 1980). For Met and Cys, additional aliquots containing 2 mg N and the internal
180 standard were pre-oxidized with 1 mL performic acid (0.9 mL of 88 % formic acid, 0.1 mL of

181 30% H_2O_2 and 5 mg phenol) for 16 h at 4°C prior to acid hydrolysis (Elkin and Griffith, 1984).
182 After hydrolysis, tube contents were filtered through Whatman 541 filter paper and filtrate was
183 diluted to 50 mL in a volumetric flask with HPLC grade H_2O. Aliquots (0.5 mL) were
184 evaporated at 60°C under constant N_2 flushing, with 3 rinses and re-evaporations with HPLC
185 grade H_2O to remove acid residues. After final evaporation, the hydrolysate was dissolved in 1
186 mL of Na diluent (Na220, Pickering Laboratories, Mountain View, CA).

187 Individual AA hydrolysates were separated using an Agilent 1100 series HPLC (Agilent
188 Technologies, Santa Clara, CA) fitted with a sodium cation exchange column (Cat. no
189 1154110T, Pikering Laboratories, Mountain View, CA) using a 4 buffer step gradient and
190 column temperature gradient. Detection of separated AA was performed at 560 nm following
191 post-column ninhydrin derivation. Standards (250 n*M*/mL) for the individual AA were prepared
192 by diluting a pure standard in sample buffer. The volume of sample and standards loaded onto
193 the column was 10 µL.

194 For Trp determination, a separate aliquot of sample containing 2 mg N was hydrolyzed with
195 1.2 g of $Ba(OH)_2$ at 110°C for the same time course as other AA on a block heater according to
196 the method of Landry and Delhaye (1992). Included in the hydrolysis was 125µL of 5-Methyl-
197 Trp (5m*M*) as an internal standard. After cooling to precipitate barium ions, an aliquot (3 µL) of
198 the hydrolysate was added to 1 mL of acetate buffer (0.07 *M* sodium acetate) an analyzed using
199 fluorescence detection (excitation = 285 nm, emission = 345 nm) after HPLC separation.

200 ***In vitro Digestibility of N and AA***

201 Microbial samples from Trials A and B were analyzed for intestinal digestibility of N and AA
202 according to the assay described by Ross et al. (2013) with minor modifications. The ruminal
203 incubation step was omitted because microbial samples were isolated from the omasum. For each

sample, 150 mg of DM was weighed in duplicate into 125 mL Erlenmeyer Flasks and 40 mL of pre-warmed ruminal buffer was added (Van Soest, 2015). Samples were then acidified to a pH of 2 with 3*M* HCl followed by addition of 2 mL of pepsin solution (282 U/mL). After 1 h of incubation at 39°C in a shaking water bath, contents of the flask were neutralized with 2 ml of 2 *M* NaOH. Ten milliliters of enzyme mixture containing trypsin, (24 mg/mL) chymotrypsin, (20 mg/mL) amylase, (50 mg/mL) and lipase (4 mg/mL) was then added to the flasks, followed by 24 h of incubation at 39°C in a shaking water bath. After incubation, flask contents were filtered on previously tared Whatman 934AH filters under vacuum. Samples were allowed to air dry, followed by drying and storage in a desiccator. Filter + residue weight was then recorded, and DM remaining on the filter was corrected for a blank carried throughout the process. Each filter was cut in half and weighed; with one half used for determination of residual N, while the other half used for AA analysis of the residual material. Determination of residual AA except Trp was performed after 24 h of hydrolysis with pre-oxidation of Met and Cys, as described previously. Insufficient sample N on the filters precluded the determination of Trp on the residues.

Calculations and Statistical Analysis

Digestibility of DM, N and individual AA was calculated as the disappearance of DM, OM, N or AA after enzymatic hydrolysis, corrected for the procedure blank. Determination of the AA concentration of microbes after multiple hydrolysis times was performed using a method similar to Rutherfurd et al. (2008) and Rutherfurd (2009). Amino acid concentration (mg/g of DM) was plotted against hydrolysis time and a non-linear equation was used to fit the curves to each plot:

$$B(t)=\frac{A_o h\left(e^{-lh}-e^{-ht}\right)}{h-l}$$

where $B(t)$ is the AA concentration at time t, h is the hydrolysis rate (proportion of bound AA hydrolyzed per hour), l is the loss rate (proportion of bound AA destroyed per hour) and A_o is the actual AA content of the protein within the sample. A_o,h and l for each sample were derived from each AA using least-squares non-linear regression with the constraints that $A_o > 0$, and $h > 0$. Rutherfurd (2009) utilized an additional term to account for free AA content (analyzed as AA determined before hydrolysis). This was not included in the current model, as free AA in bacterial and protozoal samples was considered to be negligible.

The AA profile of Trial B bacteria and protozoa determined using the different hydrolysis methods was compared using two, one-sided paired t-tests (TOST option in the TTEST procedure of SAS version 9.3; SAS Institute, Cary, NC). This procedure, common in bio-equivalence testing, allows the researcher to specify biologically relevant differences in means determined by competing methods. In this study, an AA profile difference that would theoretically alter the calculated flow of any individual AA by 6 or more g/d was considered biologically relevant for the following reasons: 1) Commercial AA products fed at 10 g/d (a common minimum feeding rate) provide approximately 6 g metabolizable AA (Whitehouse, 2016). 2) With a commonly limiting AA such as Met, a 6 g shift represents a 10% difference in the total supply of 60 g/d. To convert this 6 g/d metabolizable AA value into a relevant change in bacterial or protozoal AA profile (measured in g/100 AA) the following calculations were made: The microbial AA flowing out of the rumen of a lactating dairy cow was assumed to be approximately 1,900 g/d (Reynal et al., 2007; Fessenden, 2016). Assuming 80% of the flow is bacteria AA, and 20 % is protozoa AA (Dijkstra et al., 1998; Fessenden, 2016); this corresponds to 1,520 and 380 g/d of AA for bacteria and protozoa, respectively. Therefore, a 6 g/d change in supply corresponds to 0.4 percentage units (6 g / 1,520 g) and 1.5 percentage units (6 g / 380 g)

247 change in any individual AA in the AA profile of bacteria and protozoa, respectively. In a
248 nutrition modeling context, Fessenden (2016) saw significant improvements in CNCPS
249 predictions of AA flow when similar magnitude changes in AA profile of microbial fractions
250 were evaluated. The bio-equivalency testing framework requires thoughtful interpretation of the
251 results. Emphasis is placed on the comparison of the 90% confidence interval of the mean
252 difference and its comparison the pre-determined biologically relevant ranges defined previously
253 in this section.

254 For AA digestibility, a two-sample t-test was used to compare the digestibility estimates
255 between trials A and B. Only means from trials A and B were analyzed, as limited amount of
256 sample precluded digestibility and multiple hydrolysis time analysis for trials C and D. For all
257 analysis, n=2 for each comparison.

258 **RESULTS AND DISCUSSION**

259 ***Microbial Chemical Composition and Digestibility***

260 All donor cows were fed diets with fairly similar chemical and nutrient composition (Table
261 1). Diets were typical of the Northeastern and Midwestern US with corn silage and alfalfa silage
262 as the principal forages. Organic matter content in bacteria and protozoa was similar to values
263 obtained previously from ruminal and omasal isolates (Brito et al., 2006; 2007) although OM
264 content is strongly influenced by the isolation procedures used (Martin et al., 1994). Trial A
265 bacteria and protozoa had numerically decreased DM, OM, and N digestibility compared with
266 trial B (Table 2). This is likely due to the use of formalin in trial A vs. trial B, as formaldehyde
267 readily reacts with proteins to form products resistant to digestion (Barry, 1976). While diet and
268 animal differences between trials might also have contributed to observed differences, the
269 direction and magnitude of the difference between trial A and B for OM, DM, and N digestibility

suggests formalin treatment is a likely cause of the differences observed. Bacteria isolates had similar AA as a percent of DM, while the lower N content of bacteria from trial C (7.5 %) resulted in AA N contributing more to total N compared with bacteria from trials A and B. Protozoa from trial D had the highest AA N as a % of N. Bacterial AA N values for trials A and B were on the low end of the range (54.9 – 86.7 AA-N, % of total N) reported by Clark et al. (1992). This could be related to the site of sampling as microbes in trials A and B were isolated from omasal contents, while trials C and D were isolated from ruminal contents, which may contribute to different amounts of NAN. Volden et al. (1999b) reported diaminopimelic acid and purines were affected by diet and differed between protozoa and bacterial fractions in the rumen, and similarly, Illg and Stern (1994) noted wide ranges in non-amino N concentration between duodenal and ruminal samples.

Microbial isolates averaged 50.6 % and 49% EAA as a % of total AA for bacteria and protozoa, respectively (Table 3). Bacterial isolates from trial B had numerically increased concentrations of Lys and Met, while trials B and D protozoa also demonstrated increased Lys and Met concentrations. For NEAA, bacteria and protozoa isolations were similar among trials with the exception of Tyr, which was reduced in trials A and C. Again, the differences in microbial isolates from trial A and C for individual AA are likely due to formalin treatment. Volden et al. (1999b) reported decreased recoveries of Lys, Met, and Tyr with vs. without formaldehyde treatment in solid and liquid associated bacteria and Whitehouse et al. (1994) reported approximately 20% less Tyr in microbial samples after treatment with formaldehyde. Beyond these differences likely due to formaldehyde treatment, the AA profile of bacteria and protozoa agreed fairly well with literature reports with some exceptions. Methionine averaged 3.2 % of total AA among all samples, and was at the high end of the range reported by Clark et

al. (1992). The variability of reported AA composition is likely related more to the isolation techniques rather than true differences among microbial populations. Protozoa AA composition has been shown to remain fairly constant among sampling times (Martin et al., 1996; Volden et al., 1999a). Differences among microbial fractions (solid associated bacteria, liquid associated bacteria, and protozoa) have been well documented (Chiquette and Benchaar, 1998; Korhonen et al., 2002); however reasons for the differences are not clear. Procedures used to detach microbes report recoveries ranging from 20 % (Martín-Orúe et al., 1998) to 80 % (Whitehouse et al., 1994). This might call into question the true ability of recovered bacteria to represent the particle associated bacteria (Korhonen et al., 2002). Ultimately it is likely that differences in isolation methods are responsible for much of the reported ranges of AA composition, while a smaller portion of the variation can be considered a true difference in AA composition (Fonseca et al., 2014).

Bacteria and protozoa from Trial A demonstrated decreased digestibility (Table 4) for most AA, which again is likely related directly to formalin treatment. Total bacterial EAA digestibility averaged 74.9 and 88.0 % for trials A and B, respectively ($P = 0.01$). Protozoa AA digestibility was also likely affected by formalin treatment in trial A, however not all AA were significantly different between trials. Arginine, Leu, Val and Glu all demonstrated decreased digestibility with formalin treatment. The ability of the uN assay to detect differences in digestibility due to formalin treatment indicates the assay might be a useful evaluation tool for other protein containing feedstuffs, especially rumen protected protein supplements. Other techniques such as the mobile bag technique (Hvelplund et al., 1992) and the modified 3-step assay (Gargallo et al., 2006) rely on retention of all undigested proteins in bags, and as such, estimates of microbial digestibility of AA from those assays are of limited value due to potential for loss from the bag.

316 The in vivo nature of the mobile bag technique does ensure exposure to idealized enzymatic
317 hydrolysis conditions; however this requires extensively cannulated animals and a significant
318 amount of sample to use the method; something not easily done with isolated omasal microbial
319 samples. Heat damaged blood meal, while relatively soluble and able to pass through pores, can
320 have a very low digestible fraction (Ross et al., 2013). Alternative in vivo techniques such as the
321 precision-fed cecectomized rooster bioassay have been used in ruminant feeds (Titgemeyer et al.,
322 1990, Boucher et al., 2009), and Fonseca et al. (2014) recently applied the technique to ruminal
323 bacteria isolates. Total AA digestibility reported by Fonseca et al. (2014) averaged 76%, with a
324 range of 62 % (Cys) to 82 % (Met). The mean AA digestibility in non-formalin treated bacteria
325 in the current trial was 88%, with a range of 84 % (Tyr) to 95 % (Cys). Differences between the
326 studies are likely related to the different enzyme activities and digestive processes between these
327 two methods and the enzyme and pH differences between avian and ruminant digestion (Guerino
328 and Baumrucker, 1987; Parker, 1968; Ross et al., 2013). Storm et al., (1983) calculated a mean
329 intestinal digestibility of 85% (range of 80 to 88 %) in sheep maintained on VFA, minerals and
330 isolated ruminal microorganisms. Mathematical techniques have also been used to estimate
331 digestibility and Tas et al. (1981) utilized a regression approach to estimate true digestibility of
332 rumen bacteria at 87%, while Hvelplund and Hesselholt (1987) reported true AA digestibilities
333 between 80 and 91% for most AA using a similar approach. General agreement between the
334 previously utilized techniques in ruminants and the current application of the assay developed by
335 Ross et al. (2013) suggest that in-vitro uN determination might be useful for future studies of AA
336 digestibility in diverse supplemental protein sources of metabolizable AA.

337 ***Amino Acid Determination from Multiple Hydrolysis Times***

338 The release of individual AA in trial B bacteria and protozoa are in Figures 2-4. Extraction
339 of Ile, Met, and Val demonstrated greater release over time and thus positive slopes at time
340 points greater than 24 h and hydrolysis rate (*h*; Table 5) were lowest for these AA. Of the NEAA
341 of the protozoa, Ala, Cys and Pro demonstrated increasing concentrations of AA as hydrolysis
342 time increased. Serine concentrations of bacteria and protozoa decreased markedly after 24 h of
343 hydrolysis as indicated by a relatively high loss rate (*l*; Table 5). Overall, total AA were
344 hydrolyzed from the sample matrix at a rate of 0.415 and 0.357 mg/h for bacteria and protozoa,
345 respectively. The same least-squares non-linear regression approach has been previously
346 employed in the analysis of other AA containing compounds, including lysozyme (Darragh et al.,
347 1996), cat hair (Hendriks et al., 1998), human milk (Darragh and Moughan, 1998) and some
348 common feedstuffs (Rutherfurd, 2009). To our knowledge, no previous work has reported rumen
349 microbial AA content after multiple hydrolysis times. Rutherfurd (2009) reported similarly low *h*
350 for Ile and Val, while Ser was reported to have the highest *l* of any AA.

351 The use of multiple hydrolysis times provides some insight into the appropriateness of single
352 time point hydrolysis for AA in rumen microbial samples. While both techniques are simply
353 estimates of the theoretical unknown true AA composition, the regression method has been
354 shown to more accurately estimate the true AA profile in purified proteins (Darragh et al., 1996).
355 The AA profile determined from the regression compared with the value determined at 24 h was
356 used to establish the equivalency of the two methods in relation to biologically relevant ranges
357 (Table 6). This alternative framework of hypothesis testing requires thoughtful interpretation of
358 the results. While some AA may exhibit negligible mean differences between analysis method,
359 such as His and Thr, the interpretation of the 90% CI indicates that they are not equivalent, as the
360 CI lies outside the pre-determined rage of biologically relevant differences. Of the bacterial AA,

the 24 h time point method was determined to be not equivalent to the multiple time point hydrolysis method for every AA except Gly. The 90 % CI of the mean difference was greater than ± 1 g/100g AA for Ile, Leu, Met, and Val (Figure 5). The relatively large underestimation of Ile, Met, and Val results in an overestimation of approximately 5% for the rapidly hydrolyzed AA such as Arg, Leu, and Lys. This is similar to the results of Rutherfurd (2009), where soybean meal Ile content was underestimated by 8.4 %, followed by Val (7.0%), Ser (4.6%), and Thr (4.3%). The relatively low range in acceptable equivalence (mean difference of -0.4 to 0.4 g/g100 AA for bacteria) serves to emphasize the importance of the AA profile of bacteria on AA supply determinations.

Protozoa AA determinations between methods showed more general agreement between hydrolysis methods, largely due to the greater range in equivalence limits (mean difference of -1.5 to 1.5 g/100g AA for protozoa). Six of the 10 EAA and 6 of the 8 NEAA were deemed equivalent between methods (Table 7). Similar to the bacterial results, Ile and Met were underestimated (13.4 and 16.5 %, respectively; Figure 6) when determined with a single time point hydrolysis, resulting in over estimation of several other AA, namely Lys and Asp.

Implications for AA Predictions in Mathematical Nutritional Models

The non-equivalence of the determination methods are important to consider when developing models that rely on AA profiles of microbial protein and feedstuffs. The results from this study and the Rutherfurd (2009) data indicate that specific AA, especially Ile, Leu, Met, and Val could be underestimated in many post-ruminal AA flow studies when utilizing single time point hydrolysis between 21 and 24 h. This consideration should recognized when literature values for AA are used in development and evaluation of nutritional models that seek to accurately predict AA supply, especially those that utilize mechanistic post-absorptive sub-

models. For example, in this analysis Met was determined to contribute more to total AA than has previously been reported. Currently, the CNCPS v.6.55 uses a profile that corresponds to approximately 1.2% of microbial AA as Met (Higgs et al, 2015; Van Amburgh et al., 2015). Compared with the current analysis (4.7 % of total AA), predictions of AA supply from the model would be expected to increase more than 2 fold (assuming microbial AA accounts for 50% of total AA). Adoption of these values will likely result in a re-evaluation of many common ratios and relationships currently used to balance essential AA for lactating cattle. Given the data presented here and by Rutherfurd (2009), this might also be true for many of the EAA. The current data, especially regarding the branched-chain AA, would help explain the prediction bias for those AA observed in CNCPS v.7 despite the relatively good prediction of NAN (Higgs, 2014; Higgs et al. submitted). Overall, this analysis illustrates how sensitive nutritional models that rely on microbial AA profiles could be to errors in AA analysis, especially when a single profile accounts for a large portion of the predicted AA supply. Additionally, future studies should evaluate the use of formalin as a microbial preservative if AA analysis or digestibility is considered as an outcome. Model developers should not include any data from procedures that utilize formalin as a microbial preservative, as it will likely lead to biases and poor model evaluation.

CONCLUSIONS

Microbial composition and digestibility of individual AA are very important for the accurate predictions in many nutrition models used to feed dairy cattle. Previous literature reports have used techniques that have known limitations, while new procedures developed to address these limitations might provide better estimations of key parameters needed to properly characterize metabolizable AA supply. Multiple time point hydrolysis has been shown to improve the

407 determination of AA in feeds, and the technique has been applied here. Digestibility of AA in
408 microbial isolations were measured using a new in vitro technique. While in vivo techniques
409 might better represent the bio-physical condition of the ruminant animal, they can be expensive
410 and rely on surgically altered animals. In vitro digestibility estimates were similar to those
411 reported using previous techniques, and the assay was sensitive to formalin treatment, a process
412 known to reduce bioavailability of proteins. Methionine and branched-chain AA concentration of
413 microbial isolates was higher than previously reported and this might have implications for
414 predicting AA supply in cattle.

415 **REFERENCES**

416 AOAC International. 2005. Official methods of analysis. 18th ed. AOAC International,
417 Gaithersburg, MD.
418 Barry, T. 1976. The effectiveness of formaldehyde treatment in protecting dietary protein from
419 rumen microbial degradation. Proc Nutr Soc. 35:221-229.
420 http://dx.doi.org/10.1079/PNS19760035
421 Boucher, S. E., S. Calsamiglia, C. M. Parsons, H. H. Stein, M. D. Stern, P. S. Erickson, P. L.
422 Utterback, and C. G. Schwab. 2009. Intestinal digestibility of amino acids in rumen
423 undegradable protein estimated using a precision-fed cecectomized rooster bioassay: I.
424 Soybean meal and SoyPlus. J. Dairy Sci. 92:4489-4498.
425 http://dx.doi.org/10.3168/jds.2008-1884
426 Brito, A. F., G. A. Broderick, and S. M. Reynal. 2006. Effect of varying dietary ratios of alfalfa
427 silage to corn silage on omasal flow and microbial protein synthesis in dairy cows. J.
428 Dairy Sci. 89:3939-3953. http://dx.doi.org/10.3168/jds.S0022-0302(06)72436-5

429 Brito, A. F., G. A. Broderick, and S. M. Reynal. 2007. Effects of different protein supplements
430 on omasal nutrient flow and microbial protein synthesis in lactating dairy cows. J. Dairy
431 Sci. 90:1828-1841. http://dx.doi.org/10.3168/jds.2006-559 doi:10.3168/jds.2006-559
432 Chiquette, J. and C. Benchaar. 1998. Effect of diet and probiotic addition on chemical
433 composition of free or particle-associated bacterial populations of the rumen. Can J.
434 Anim. Sci. 78:115-120. http://dx.doi.org/10.4141/A97-043
435 Clark, J. H., T. H. Klusmeyer, and M. R. Cameron. 1992. Microbial protein synthesis and flows
436 of nitrogen fractions to the duodenum of dairy cows. J. Dairy Sci. 75:2304-2323.
437 http://dx.doi.org/10.3168/jds.S0022-0302(92)77992-2
438 Darragh, A. J., D. J. Garrick, P. J. Moughan, and W. H. Hendriks. 1996. Correction for amino
439 acid loss during acid hydrolysis of a purified protein. Anal. Biochem. 236:199-207.
440 http://dx.doi.org/10.1006/abio.1996.0157
441 Darragh, A. J. and P. J. Moughan. 1998. The amino acid composition of human milk corrected
442 for amino acid digestibility. Br. J. Nutr. 80:25-34.
443 http://dx.doi.org/10.1017/S0007114598001731
444 Darragh, A. J. and P. J. Moughan. 2005. The effect of hydrolysis time on amino acid analysis. J.
445 AOAC Int. 88:888-893.
446 Denton, B. L., L. E. Diese, J. L. Firkins, and T. J. Hackmann. 2015. Accumulation of reserve
447 carbohydrate by rumen protozoa and bacteria in competition for glucose. Appl. Environ.
448 Microbiol. 81:1832-1838. http://dx.doi.org/10.1128/AEM.03736-14.
449 Dijkstra, J., J. France, and S. Tamminga. 1998. Quantification of the recycling of microbial
450 nitrogen in the rumen using a mechanistic model of rumen fermentation processes. J.
451 Agric. Sci. 130:81-94.

452 Elkin, R. and J. Griffith. 1984. Hydrolysate preparation for analysis of amino acids in sorghum
453 grains: effect of oxidative pretreatment. J. AOAC 68:1117-1121.
454 Fessenden, S. W. 2016. Amino acid supply in dairy cattle. Ph.D. Dissertation. Cornell Univ.,
455 Ithaca, NY. https://ecommons.cornell.edu/handle/1813/45365
456 Fonseca, A., S. Fredin, L. Ferraretto, C. Parsons, P. Utterback, and R. Shaver. 2014. Short
457 communication: Intestinal digestibility of amino acids in fluid-and particle-associated
458 rumen bacteria determined using a precision-fed cecectomized rooster bioassay. J. Dairy
459 Sci. 97:3855-3859. http://dx.doi.org/10.3168/jds.2013-7880
460 Gargallo, S., S. Calsamiglia, and A. Ferret. 2006. Technical note: A modified three-step in vitro
461 procedure to determine intestinal digestion of proteins. J. Anim. Sci. 84:2163-2167.
462 http://dx.doi.org/10.2527/jas.2004-704
463 Guerino, F. and C. R. Baumrucker. 1987. Methionine and lysine uptake by cattle small intestine
464 *in vitro*. J. Anim. Sci. 65:619-629.
465 Gehrke, C. W., L. Wall Sr, J. Absheer, F. Kaiser, and R. Zumwalt. 1985. Sample preparation for
466 chromatography of amino acids: acid hydrolysis of proteins. J. AOAC 68:811-821.
467 Hendriks, W., M. Tarttelin, and P. Moughan. 1998. The amino acid composition of cat (*Felis*
468 *catus*) hair. Anim. Sci. 67:165-170. http://dx.doi.org/10.1017/S1357729800009905
469 Higgs, R. 2014. Development of a dynamic rumen and gastro-intestinal model in the Cornell Net
470 Carbohydrate and Protein System to predict the nutrient supply and requirements of dairy
471 cattle. Ph.D. Dissertation. Cornell Univ., Ithaca, NY.
472 Higgs, R., L. Chase, D. Ross, and M. Van Amburgh. 2015. Updating the Cornell Net
473 Carbohydrate and Protein System feed library and analyzing model sensitivity to feed
474 inputs. J. Dairy Sci. 98:6340-6360. http://dx.doi.org/10.3168/jds.2015-9379.

475 Huhtanen, P., P. G. Brotz, and L. D. Satter. 1997. Omasal sampling technique for assessing
476 fermentative digestion in the forestomach of dairy cows. J. Anim. Sci. 75:1380-1392.
477 http://dx.doi.org/10.2527/1997.7551380x.
478 Hvelplund, T. and M. Hesselholt. 1987. Digestibility of individual amino acids in rumen
479 microbial protein and undegraded dietary protein in the small intestine of sheep. Acta
480 Agric. Scand. 37:469-477. http://dx.doi.org/10.1080/00015128709436578
481 Hvelplund, T., M. R. Weisbjerg, and L. S. Andersen. 1992. Estimation of the true digestibility of
482 rumen undegraded dietary protein in the small intestine of ruminants by the mobile bag
483 technique. Acta Agric. Scand. A Anim. Sci. 42:34-39.
484 http://dx.doi.org/10.1080/09064709209410106
485 Illg, D. and M. Stern. 1994. In vitro and in vivo comparisons of diaminopimelic acid and purines
486 for estimating protein synthesis in the rumen. Anim. Feed Sci. Technol. 48:49-55.
487 http://dx.doi.org/10.1016/0377-8401(94)90111-2. Vol. V.
488 Keller, P. J. 1968. Pancreatic proteolytic enzymes. American Physiology Society. Handbook of
489 Physiology. Section 6: Alimentary Canal. 122:2605-2628. Wash. D.C.
490 Korhonen, M., S. Ahvenjärvi, A. Vanhatalo, and P. Huhtanen. 2002. Supplementing barley or
491 rapeseed meal to dairy cows fed grass-red clover silage: II. Amino acid profile of
492 microbial fractions. J. Anim. Sci. 80:2188-2196.
493 http://dx.doi.org/10.2527/2002.8082188x
494 Landry, J. and S. Delhaye. 1992. Simplified procedure for the determination of tryptophan of
495 foods and feedstuffs from barytic hydrolysis. J. Agric. Food Chem. 40:776-779.
496 Martín-Orúe, S. M., J. Balcells, F. Zakraoui, and C. Castrillo. 1998. Quantification and chemical
497 composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of

498 detachment procedure. Anim. Feed Sci. Technol. 71:269-282.
499 http://dx.doi.org/10.1016/S0377-8401(97)00156-9
500 Martin, C., L. Bernard, and B. Michalet-Doreau. 1996. Influence of sampling time and diet on
501 amino acid composition of protozoal and bacterial fractions from bovine ruminal
502 contents. J. Anim. Sci. 74:1157-1163. http://dx.doi.org/10.2527/1996.7451157x
503 Martin, C., A. G. Williams, and B. Michalet-Doreau. 1994. Isolation and characteristics of the
504 protozoal and bacterial fractions from bovine ruminal contents. J. Anim. Sci. 72:2962-
505 2968. http://dx.doi.org/10.2527/1994.72112962x
506 Mason, V. C., S. Bech-Andersen, and M. Rudemo. 1980. Hydrolysate preparation for amino acid
507 determinations in feed constituents. Z Tierphysiol Tierernahr Futtermittelkd. 43:146-164.
508 http://dx.doi.org/10.1111/j.1439-0396.1980.tb00618.x
509 National Research Council. 2001. Nutrient requirements of dairy cattle. 7th revised ed. National
510 Academy Press, Washington, DC.
511 Recktenwald, E. B., D. A. Ross, S. W. Fessenden, C. J. Wall , and M. E. Van Amburgh. 2013.
512 Urea-N recycling in lactating dairy cows fed diets with 2 different levels of dietary crude
513 protein and starch with or without monensin. J. Dairy Sci. 97:1611–1622.
514 http://dx.doi.org/10.3168/jds.2013-7162
515 Recktenwald, E. B. 2010. Urea N recycling and its utilization by ruminal microbial populations
516 in lactating dairy cattle. Ph.D. Dissertation. Cornell Univ., Ithaca, NY.
517 http://hdl.handle.net/1813/17757.
518 Reynal, S. M., G. A. Broderick, S. Ahvenjärvi, and P. Huhtanen. 2003. Effect of feeding protein
519 supplements of differing degradability on omasal flow of microbial and undegraded

protein. J. Dairy Sci. 86:1292-1305. http://dx.doi.org/10.3168/jds.S0022-0302(03)73713-8.

Reynal, S. M. and G. A. Broderick. 2005. Effect of dietary level of rumen-degraded protein on production and nitrogen metabolism in lactating dairy cows. J. Dairy Sci. 88:4045-4064. http://dx.doi.org/10.3168/jds.S0022-0302(05)73090-3.

Reynal, S. M., I. R. Ipharraguerre, M. Lineiro, A. F. Brito, G. A. Broderick, and J. H. Clark. 2007. Omasal flow of soluble proteins, peptides, and free amino acids in dairy cows fed diets supplemented with proteins of varying ruminal degradabilities. J. Dairy Sci. 90:1887-1903. http://dx.doi.org/10.3168/jds.2006-158.

Ross, D. A. 2013. Methods to analyze feeds for nitrogen fractions and digestibility for ruminants with application for the CNCPS. Ph.D. dissertation. Cornell University, Ithaca, NY. https://ecommons.cornell.edu/handle/1813/33993

Ross, D. A., M. Gutierrez-Botero, and M. E. Van Amburgh. 2013. Development of an in-vitro intestinal digestibility assay for ruminant feeds. Pages 190-202 in Proc. Cornell Nutrition Conference, Syracuse, NY. Cornell University, Ithaca, NY.

Ross, D. A. 2004. Amino acid composition of ruminant feeds and feed fractions and evaluation of the methods used to obtain the insoluble and true precipitate protein fractions of feedstuffs. M.S. Thesis. Cornell University. Ithaca, NY

Rossi, M. F., I. Martinele and M. D'Agosto. 2013. Quantitative and differential analysis of ciliate protozoa in rumen content samples filtered before and after fixation. R. Bras. Zootec. 42:831-834. http://dx.doi.org/10.1590/S1516-35982013001100010

Rutherfurd, S. M. 2009. Accurate determination of the amino acid content of selected feedstuffs. Int. J. Food Sci. Nutr. 60:53-62. http://dx.doi.org/10.1080/09637480802269957

543 Rutherfurd, S. M., P. J. Moughan, D. Lowry, and C. G. Prosser. 2008. Amino acid composition
544 determined using multiple hydrolysis times for three goat milk formulations. Int. J. Food
545 Sci. Nutr. 59:679-690. http://dx.doi.org/10.1080/09637480701705424
546 Storm, E., D. S. Brown, and E. R. Ørskov. 1983. The nutritive value of rumen micro-organisms
547 in ruminants 3. The digestion of microbial amino and nucleic acids in, and losses of
548 endogenous nitrogen from, the small intestine of sheep. Br. J. Nutr. 50:479-485.
549 Sylvester, J. T., S. K. Karnati, Z. Yu, M. Morrison, and J. L. Firkins. 2004. Development of an
550 assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. J. Nutr.
551 134:3378-3384.
552 Sylvester, J. T., S. K. R. Karnati, Z. Yu, C. J. Newbold, and J. L. Firkins. 2005. Evaluation of a
553 real-time PCR assay quantifying the ruminal pool size and duodenal flow of protozoal
554 nitrogen. J. Dairy Sci. 88:2083-2095. http://dx.doi.org/10.3168/jds.S0022-
555 0302(05)72885-X
556 Tas, M., R. Evans, and R. Axford. 1981. The digestibility of amino acids in the small intestine of
557 the sheep. Br. J. Nutr. 45:167-174. https://doi.org/10.1079/BJN19810089
558 Titgemeyer, E., N. Merchen, Y. Han, C. Parsons, and D. Baker. 1990. Assessment of intestinal
559 amino acid availability in cattle by use of the precision-fed cecectomized rooster assay. J.
560 Dairy Sci. 73:690-693. http://dx.doi.org/10.3168/jds.S0022-0302(90)78721-8
561 Van Amburgh, M., E. Collao-Saenz, R. Higgs, D. Ross, E. Recktenwald, E. Raffrenato, L.
562 Chase, T. Overton, J. Mills, and A. Foskolos. 2015. The Cornell Net Carbohydrate and
563 Protein System: Updates to the model and evaluation of version 6.5. J. Dairy Sci.
564 98:6361-6380. http://dx.doi.org/10.3168/jds.2015-9378.

565 Van Soest, P. J. 2015. The Detergent System for Analysis of Foods and Feeds. Cornell
566 University, Ithaca, NY. ISBN 9781630951344.
567 Volden, H. and O. M. Harstad. 1998. Amino acid composition of bacteria harvested from the
568 rumen of dairy cows fed three diets differing in protein content and rumen protein
569 degradability at two levels of intake. Acta Agric. Scand. A Anim. Sci. 48:210-215.
570 http://dx.doi.org/10.1080/09064709809362422.
571 Volden, H., O. M. Harstad, and L. T. Mydland. 1999a. Amino acid content and profile of
572 protozoal and bacterial fractions isolated from ruminal contents of lactating dairy cows
573 fed diets differing in nitrogen supplementation. Acta Agric. Scand. A Anim. Sci. 49:245-
574 250. http://dx.doi.org/10.1080/090647099424006.
575 Volden, H., L. T. Mydland, and O. M. Harstad. 1999b. Chemical composition of protozoal and
576 bacterial fractions isolated from ruminal contents of dairy cows fed diets differing in
577 nitrogen supplementation. Acta Agric. Scand. A Anim. Sci. 49:235-244.
578 http://dx.doi.org/10.1080/090647099423999
579 Whitehouse, N., V. Olson, C. Schwab, W. Chesbrot, K. Cunningham, and T. Lykos. 1994.
580 Improved techniques for dissociating particle-associated mixed ruminal microorganisms
581 from ruminal digesta solids. J. Anim. Sci 72:1335-1343.
582 http://dx.doi.org/10.2527/1994.7251335x.
583 Whitehouse, N. L. 2016. Using the plasma free amino acid dose response method to determine
584 metabolizable protein concentrations of lysine and methionine in rumen protected
585 supplements. PhD dissertation. Univ. New Hampshire, 2016.
586 http://pqdtopen.proquest.com/pubnum/10248580.html?FMT=AI.
587

Table 1. Donor cow diet ingredient and chemical composition, intake, and milk production

	Trial[1]			
Item	A	B	C	D[2]
Number of isolations in each sample:	72	108	NA	8
Diet ingredient composition				
Corn silage	44.0	28.6	45.6	45.3
Haycrop silage	12.0	22.9	-	13.8
Wheat straw	-	-	2.1	-
Corn meal	12.0	28.6	11.1	12.5
Barley grain, ground	-	-	6.2	-
Soybean meal	-	-	4.5	8.6
Canola meal	-	9.2	-	-
Rumen protected soybean meal	8.2	-	3.8	2.1
Commercial fermentation byproduct	1.5	-	-	-
Corn distillers	-	-	-	3.8
Cottonseed	-	-	8.4	6.4
Wheat middlings	3.4	-	-	2.8
Soybean hulls	5.8	5.5	-	-
Citrus pulp	3.3	1.0	7.3	-
Sugar	-	-	2.2	-
Molasses	0.9	-	-	-
Fatty acid supplement	1.2	0.6	-	1.0
Blood meal	1.7	1.4	1.1	-
Minerals, vitamins, and additives	6.2	2.2	7.7	3.7
Diet chemical composition				
OM, % of DM	93.9	92.6	92.5	91.8
CP, % of DM	16.0	16.9	14.8	16.3
Soluble protein, % of CP	35.7	43.7	31.9	34.1
RDP, % of CP[3]	51.9	59.8	54.3	62.0
aNDFom, % of DM	31.1	30.6	33.6	32.1
ADF, % of DM	19.8	20.5	NA	20.2
ADL, % of DM	3.0	3.4	1.9	3.1
Sugars, % of DM	5.4	3.5	5.4	2.8
Starch, % of DM	27.6	27.9	25.4	26.7
Ether extract, % of DM	4.9	3.9	4.5	5.6
ME, Mcal/kg[3]	2.5	2.6	2.7	2.6
Cattle intake and production				
Dry matter intake, kg/d	27.3	26.1	23.8	NA
Milk production, kg/d	41.7	41.6	30.9	NA

[1]Trial A: Fessenden (2016); Trial B: Foskolos et al., (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2013); Trial D: T. Hackmann, personal communication
[2]Chemical composition estimated using CNCPS v. 6.55 using diet ingredient composition
[3]Metabolizable energy predicted using CNCPS v. 6.55

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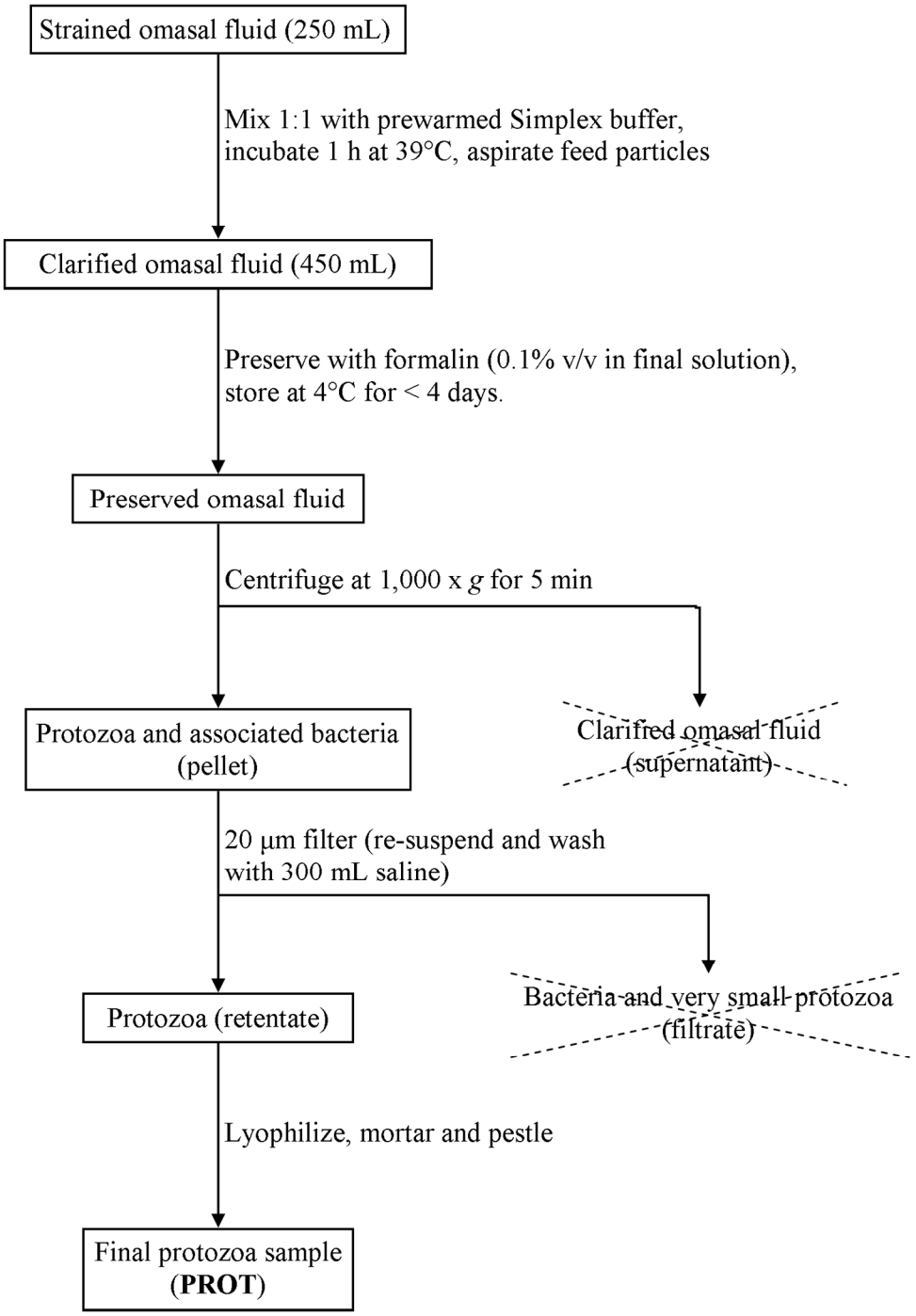
589



590

591 **Figure 1**. Flowchart for preparation of protozoa isolates in trials A and B. Fractions discarded

592 are crossed out. Trial B isolation differed only in the omission of the formalin preservation step.

593

Table 2. Chemical composition and intestinal digestibility of bacteria and protozoa isolates

	Trial[1]			
Item	A	B	C	D[2]
Bacteria				-
OM, % of DM	83.9	92.1	90.1	-
N, % of DM	8.1	8.9	7.5	-
uN, % of total N[2]	36.4	15.1	-	-
AA, % of DM	28.2	34.3	32.5	
AA N, % of N	47.3	51.8	57.8	-
DM digestibility, %	53.6	69.0	-	-
OM digestibility, %	68.2	81.9	-	-
N digestibility, %	63.6	84.9	-	-
Protozoa				
OM, % of DM	87.0	85.0	90.8	93.8
N, % of DM	8.3	8.2	8.1	8.4
uN, % total N[2]	46.7	24.7	-	-
AA, % of DM	28.4	31.7	37.4	45.3
AA N, % of N	53.2	53.7	65.9	71.3
DM digestibility, %	48.7	61.9	-	-
OM digestibility, %	65.9	88.4	-	-
N digestibility, %	53.3	75.3	-	-

[1]Trial A: Fessenden (2016); Trial B: Foskolos et al., (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2013); Trial D: T. Hackmann, personal communication

[2]intestinally unavailable N as determined by the procedure of Ross et al. (2013)

594

Table 3. Amino acid profile (% of total AA) of microbial isolates using a 24 h hydrolysis time.

	Bacteria AA				Protozoa AA			
	Trial[1]				Trial[1]			
Item	A	B	C	D	A	B	C	D
Essential AA								
Arg	5.4	5.0	5.0	-	5.5	5.4	5.4	4.7
His	2.1	2.1	2.0	-	2.9	2.5	2.3	2.0
Ile	5.0	4.0	4.9	-	4.7	3.8	5.7	5.5
Leu	4.8	5.6	6.8	-	5.5	6.1	4.6	4.2
Lys	4.7	7.5	4.8	-	5.7	8.8	5.3	10.2
Met	3.3	4.5	2.6	-	2.7	3.1	2.1	3.8
Phe	6.6	6.0	6.8	-	7.4	6.5	7.3	7.6
Thr	6.3	5.7	5.4	-	5.4	4.8	6.1	4.7
Trp	5.7	5.5	5.3	-	4.6	4.5	3.1	1.4
Val	6.7	5.9	5.6	-	5.7	4.7	4.7	4.7
Total EAA	50.8	51.9	49.3	-	50.1	50.2	46.5	48.8
Non-essential AA								
Ala	7.9	6.9	6.7	-	5.9	5.1	4.7	4.0
Asp	12.2	11.1	8.8	-	11.8	10.8	12.1	11.6
Cys	1.4	1.5	1.1	-	2.0	2.1	1.7	2.2
Glu	12.4	11.3	13.0	-	14.0	13.6	15.2	13.6
Gly	5.7	4.9	5.2	-	4.7	4.5	4.3	4.0
Pro	2.1	2.0	6.9	-	2.7	3.0	7.9	5.6
Ser	5.5	4.6	5.4	-	5.3	5.4	5.5	4.2
Tyr	2.0	6.0	3.5	-	3.5	5.4	2.0	6.1
Total NEAA	49.2	48.1	50.7	-	49.9	49.8	53.5	51.2

[1]Trial A: Fessenden (2016); Trial B: Foskolos et al., (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2013); Trial D: T. Hackmann, personal communication

595

Table 4. Intestinal digestibility (% of AA) of AA of omasal bacteria and protozoa

	Bacteria				Protozoa			
	Trial[1]				Trial[1]			
Item	A	B	SEM	*P*	A	B	SEM	*P*
Essential AA								
Arg	74.0	88.2	0.9	0.03	69.0	89.0	0.4	0.01
His	78.4	90.7	1.9	0.03	69.7	70.9	11.9	0.93
Ile	76.8	88.9	0.6	0.00	75.3	85.7	3.0	0.08
Leu	78.5	92.2	1.5	0.01	74.3	92.5	1.1	0.01
Lys	75.5	91.0	1.8	0.02	68.2	77.5	12.6	0.54
Met	80.7	88.8	1.0	0.07	78.4	90.1	0.9	0.17
Phe	69.0	83.2	1.2	0.01	67.5	79.7	2.7	0.06
Thr	76.3	89.8	1.1	0.01	73.5	79.2	9.3	0.61
Trp	-	-	-	-	-	-	-	-
Val	69.9	88.0	4.0	0.05	63.2	83.3	3.8	0.04
Total EAA	74.9	88.0	1.4	0.01	70.6	82.8	4.4	0.11
Non-essential AA								
Ala	72.5	87.2	2.2	0.02	67.5	80.1	6.6	0.20
Asp	77.5	90.8	1.2	0.01	75.8	86.3	5.0	0.18
Cys	82.1	94.8	0.7	0.05	89.3	93.6	1.7	0.15
Glu	68.5	85.2	3.6	0.04	67.5	87.7	0.8	0.01
Gly	69.4	85.8	3.7	0.05	63.5	72.8	13.4	0.56
Pro	77.9	88.4	1.4	0.10	78.1	80.7	4.7	0.66
Ser	75.0	89.3	1.3	0.01	71.9	56.7	33.6	0.70
Tyr	34.0	84.3	0.4	0.00	55.9	73.8	4.5	0.06
Total NEAA	71.6	87.5	1.9	0.02	70.2	80.2	7.6	0.32
Total AA	73.3	87.8	1.7	0.01	70.4	81.6	6.0	0.21

[1]Trial A: Fessenden (2016); Trial B: Foskolos et al., (unpublished data); Trial C: Recktenwald (2010) and Recktenwald et al. (2013); Trial D: T. Hackmann, personal communication. n=2 for each comparison

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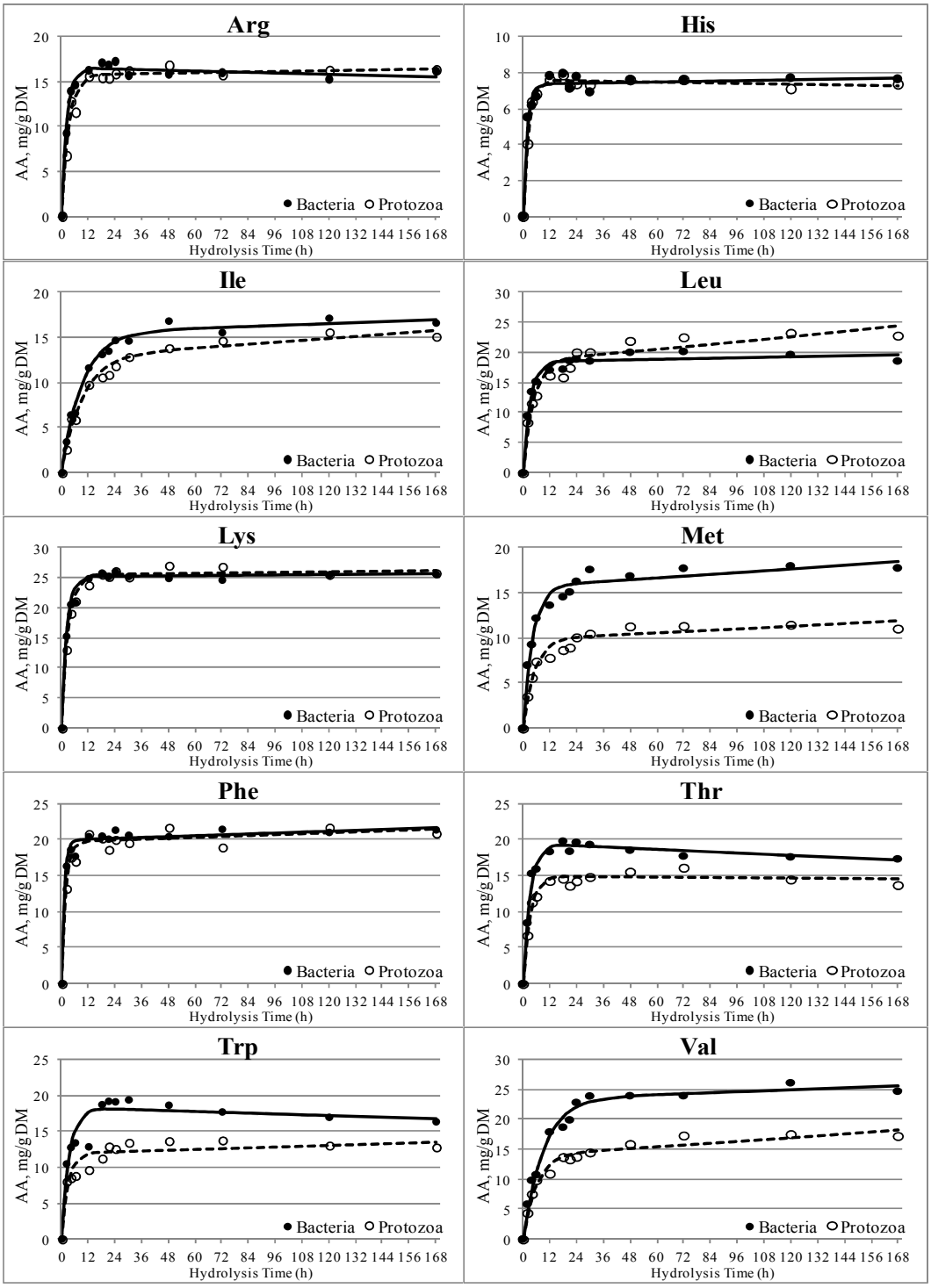
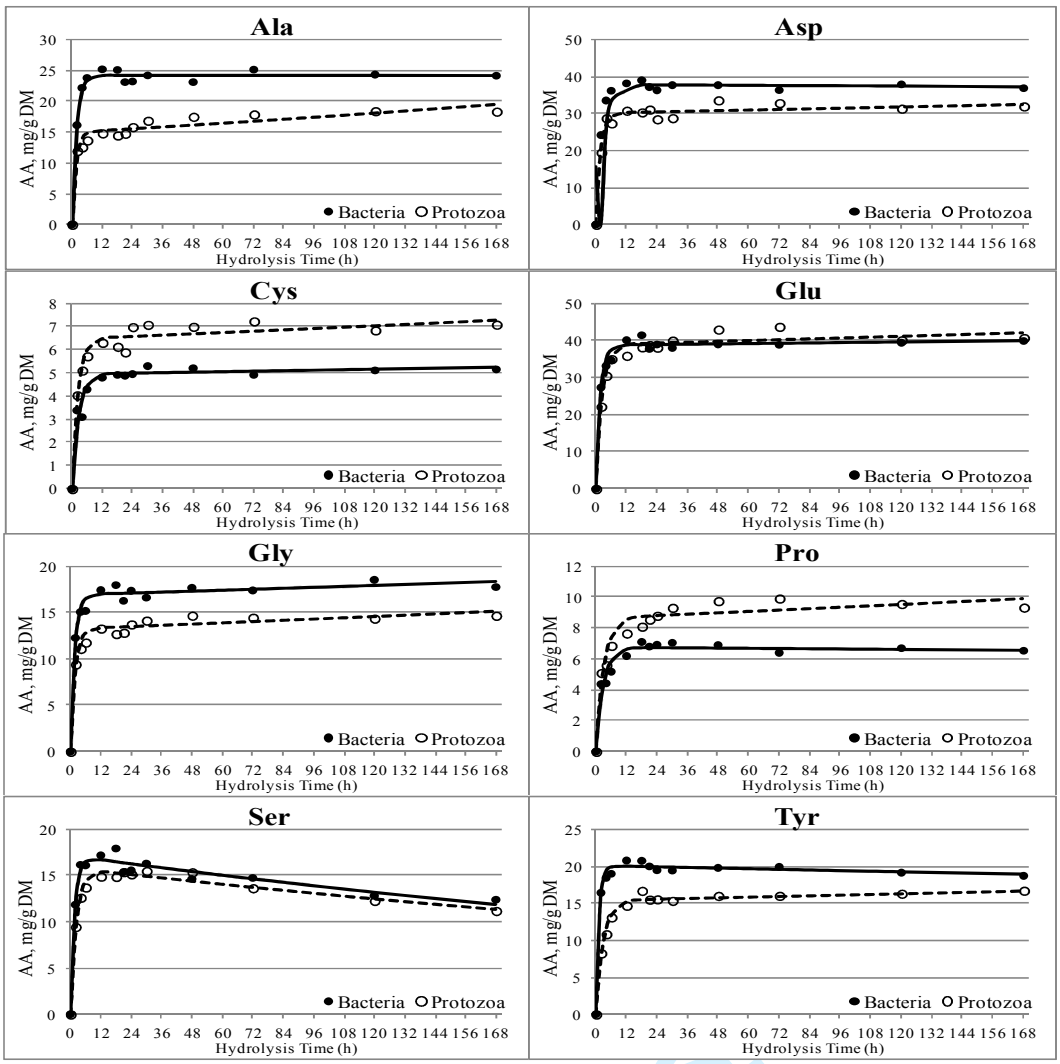


598

599 **Figure 2.** Effect of hydrolysis time (h) on content of essential AA (mg/g DM) from freeze dried
600 isolations of omasal bacteria (●) and protozoa (○) from Trial B. The mean of each time point (2
601 replicates each) is plotted against the least squared regression line for each respective dataset.



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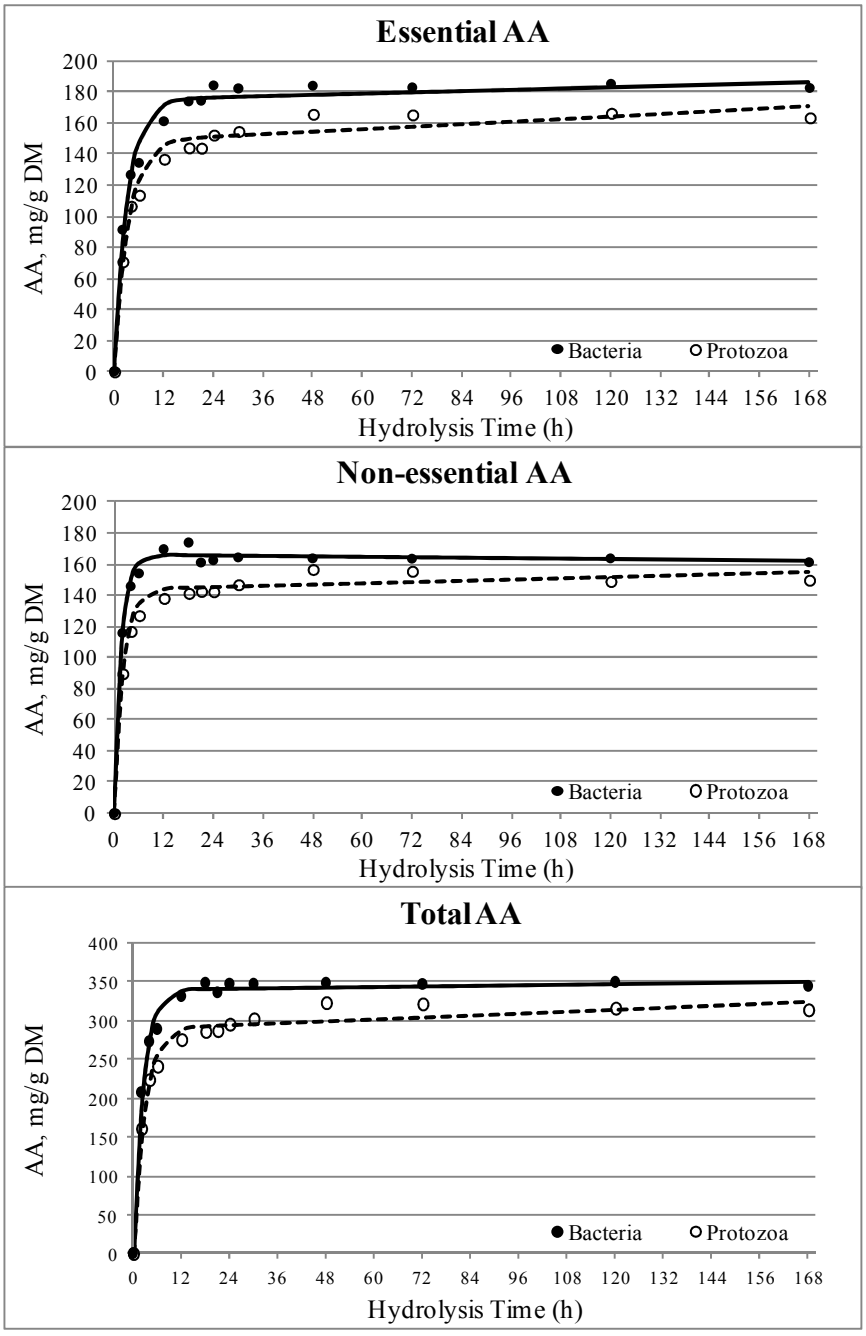
603 **Figure 3**. Effect of hydrolysis time (h) on content of non-essential AA (mg/g DM) from freeze

604 dried isolations of omasal bacteria (●) and protozoa (○) from Trial B. The mean of each time

605 point (2 replicates each) is plotted against the least squared regression line for each respective

606 dataset.



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608 **Figure 4.** Effect of hydrolysis time (h) on content of essential, non-essential, and total AA (mg/g
609 DM) from freeze dried isolations of omasal bacteria (●) and protozoa (○) from Trial B. The
610 mean of each time point (2 replicates each) is plotted against the least squared regression line for
611 each respective dataset.

Table 5. Rate of hydrolysis (h)[1] and loss (l)[2] for individual and total AA from omasal bacteria and protozoa isolates from Trial B. Values are presented as the mean (SEM).

	Bacteria		Protozoa	
Item	h (h^{-1})	l (h^{-1})	h (h^{-1})	l (h^{-1})
Essential AA				
Arg	0.414 (0.045)	0.00039 (0.00078)	0.303 (0.027)	-0.00025 (0.00001)
His	0.577 (0.222)	-0.00027 (0.00048)	0.409 (0.110)	0.00026 (0.00038)
Ile	0.107 (0.037)	-0.00054 (0.00080)	0.106 (0.007)	-0.00122 (0.00034)
Leu	0.323 (0.019)	-0.00037 (0.00086)	0.217 (0.002)	-0.00162 (0.00060)
Lys	0.421 (0.017)	-0.00012 (0.00034)	0.333 (0.026)	-0.00016 (0.00051)
Met	0.234 (0.079)	-0.00097 (0.00008)	0.198 (0.100)	-0.00112 (0.00041)
Phe	0.782 (0.193)	-0.00050 (0.00108)	0.529 (0.286)	-0.00052 (0.00086)
Thr	0.323 (0.056)	0.00077 (0.00007)	0.318 (0.030)	0.00015 (0.00042)
Trp	0.283 (0.196)	0.00054 (0.00090)	0.332 (0.303)	-0.00072 (0.00122)
Val	0.312 (0.054)	-0.00054 (0.00055)	0.169 (0.021)	-0.00153 (0.00003)
Total EAA	0.303 (0.009)	-0.00038 (0.00022)	0.287 (0.002)	-0.00084 (0.00033)
Non-essential AA				
Ala	0.571 (0.183)	0.00001 (0.00063)	0.658 (0.132)	-0.00156 (0.00100)
Asp	0.523 (0.057)	0.00014 (0.00039)	0.548 (0.168)	-0.00044 (0.00077)
Cys	0.377 (0.035)	-0.00035 (0.00005)	0.429 (0.125)	-0.00070 (0.00023)
Glu	0.550 (0.027)	-0.00019 (0.00005)	0.397 (0.060)	-0.00048 (0.00118)
Gly	0.599 (0.174)	-0.00049 (0.00020)	0.541 (0.030)	-0.00081 (0.00009)
Pro	0.324 (0.115)	0.00018 (0.00021)	0.304 (0.014)	-0.00081 (0.00045)
Ser	0.622 (0.097)	0.00218 (0.00049)	0.418 (0.011)	0.00198 (0.00079)
Tyr	0.804 (0.254)	0.00036 (0.00024)	0.328 (0.476)	-0.00046 (0.00038)
Total NEAA	0.568 (0.112)	0.00014 (0.00024)	0.447 (0.107)	-0.00045 (0.00075)
Total AA	0.415 (0.048)	-0.00018 (0.00021)	0.357 (0.037)	-0.00067 (0.00054)

[1]proportion of bound AA hydrolyzed per hour.
[2]proportion of bound AA destroyed per hour.

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Table 6. Comparison of the AA composition (g/100 g AA) of omasal bacteria from trial B[1] determined using multiple hydrolysis time point or single hydrolysis time point methods.

	Method				90 % CI		
AA	Single	Multiple	S - M	SED[2]	Lower	Upper	EQ
Essential AA							
Arg	5.00	4.73	0.27	0.03	0.06	0.48	No
His	2.12	2.11	0.01	0.14	-0.85	0.86	No
Ile	4.05	4.62	-0.58	0.46	-3.46	2.31	No
Leu	5.60	5.32	0.28	0.26	-1.35	1.91	No
Lys	7.54	7.17	0.37	0.04	0.11	0.63	No
Met	4.49	4.63	-0.14	0.36	-2.41	2.13	No
Phe	6.00	5.77	0.23	0.09	-0.31	0.77	No
Thr	5.49	5.53	-0.04	0.10	-0.69	0.60	No
Trp	5.97	5.77	0.20	0.03	-0.01	0.41	No
Val	5.92	6.32	-0.41	0.32	-2.41	1.60	No
Non-essential AA							
Ala	6.88	6.94	-0.06	0.21	-1.36	1.24	No
Asp	11.06	10.79	0.28	0.03	0.05	0.50	No
Cys	1.45	1.40	0.05	0.07	-0.40	0.51	No
Glu	11.26	11.06	0.21	0.58	-3.45	3.86	No
Gly	4.86	4.83	0.02	0.02	-0.10	0.15	Yes
Pro	2.03	1.91	0.12	0.16	-0.89	1.12	No
Ser	4.58	4.89	-0.31	0.44	-3.06	2.44	No
Tyr	5.71	5.65	0.06	0.13	-0.76	0.89	No

[1]Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.
[2]Standard error of the difference.
[2]Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -0.4 to 0.4 g/100g of AA.

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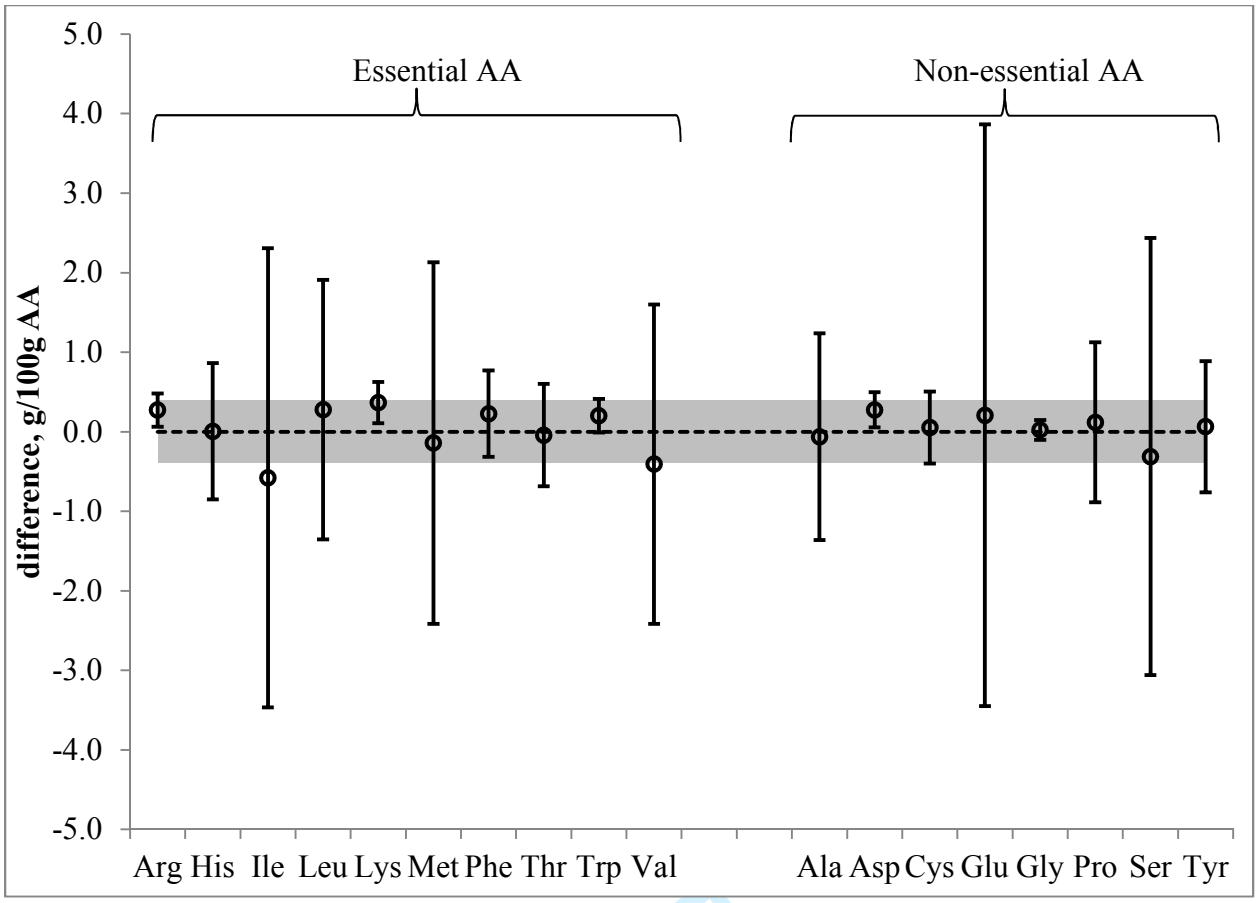

Figure 5. Equivalency chart for difference in AA composition (g/100 g AA) of Trial B bacteria determined using multiple vs. single time point hydrolysis methods. Open circles (○) represent the mean difference and error bars represent the 90% confidence interval around the mean difference. Shaded region represents equivalency defined as -0.4 to 0.4 g/100 g bacterial AA.

Table 7. Comparison of the AA composition (g/100 g AA) of omasal protozoa from trial B[1] determined using multiple vs. single time point hydrolysis methods.

	Method				90 % CI		
AA	Single	Multiple	S - M	SED[2]	Lower	Upper	EQ[3]
Essential AA							
Arg	5.35	5.26	0.09	0.15	-0.84	1.03	Yes
His	2.53	2.52	0.01	0.01	-0.03	0.05	Yes
Ile	3.80	4.39	-0.59	0.06	-0.94	-0.24	Yes
Leu	6.11	6.25	-0.14	0.41	-2.70	2.42	No
Lys	8.81	8.55	0.26	0.06	-0.10	0.62	Yes
Met	3.14	3.77	-0.62	0.47	-3.58	2.34	No
Phe	6.49	6.58	-0.08	0.24	-1.61	1.45	No
Thr	5.41	5.34	0.07	0.03	-0.13	0.26	Yes
Trp	4.76	4.95	-0.19	0.27	-1.90	1.52	No
Val	4.65	4.75	-0.10	0.04	-0.38	0.18	Yes
Non-essential AA							
Ala	5.14	5.03	0.11	0.07	-0.31	0.53	Yes
Asp	10.85	10.14	0.71	0.47	-2.27	3.69	No
Cys	2.06	2.16	-0.09	0.01	-0.15	-0.04	Yes
Glu	13.56	13.03	0.52	0.04	0.30	0.74	Yes
Gly	4.48	4.41	0.07	0.01	0.03	0.12	Yes
Pro	2.99	2.89	0.10	0.10	-0.52	0.71	Yes
Ser	5.36	5.28	0.08	0.03	-0.14	0.29	Yes
Tyr	4.50	4.70	-0.20	0.34	-2.32	1.92	No

[1]Trial B: Foskolos et al., (unpublished data). n=2 for all comparisons.
[2]Standard error of the difference.
[3]Equivalence determined from 2 one-sided paired t-tests. Methods deemed to be equivalent if 90% CI falls within defined equivalency of -1.5 to 1.5 g/100g of AA.

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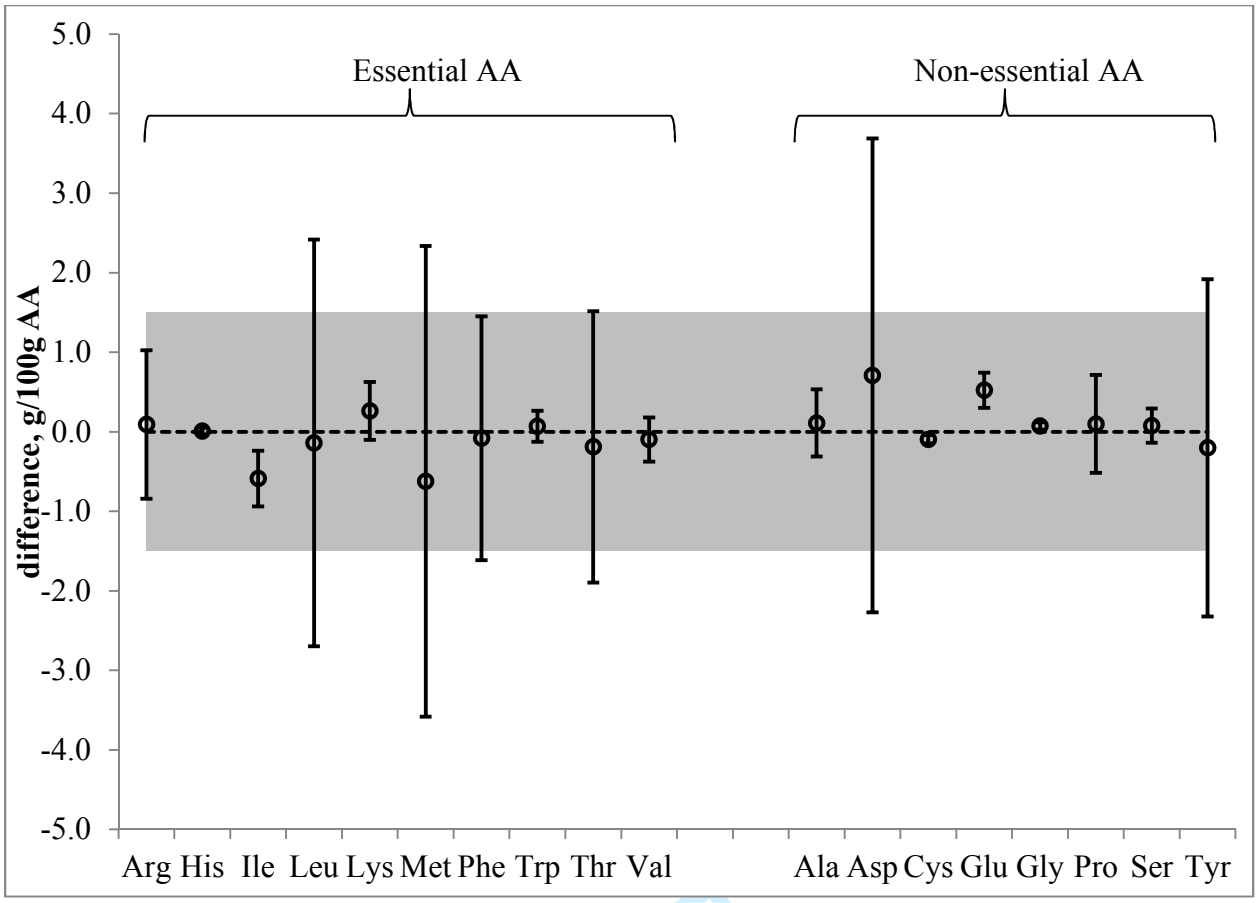

Figure 6. Equivalency chart for difference in AA composition (g/100 g AA) of Trial B protozoa determined using multiple vs. single time point hydrolysis methods. Open circles (○) represent the mean difference and error bars represent the 90% confidence interval around the mean difference. Shaded region represents equivalency defined as -1.5 to 1.5 g/100 g protozoal AA.